

Heavy-Metal-Induced Reactive Oxygen Species: Phytotoxicity and Physicochemical Changes in Plants

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1 Introduction

Environmental contamination by hazardous environmental pollutants is a widespread and increasingly serious problem confronting society, scientists, and regulators worldwide (Debenest et al. 2010; Hajeb et al. 2011; Nanthi and Bolan 2012; Shahid et al. 2013a). Among these pollutants, the heavy metals, are a loosely-defined group of elements that are similar in that they all exhibit metallic properties, and have atomic masses >20 (excluding the alkali metals) and specific gravities >5 (Rascio and Navari-Izzo 2011). This group mainly includes transition metals, some metalloids, and the lanthanides and actinides. Heavy metals can be toxic to plants, animals and humans, even at very low concentrations. Heavy metals are natural components of the earth's crust and are present in different concentrations at different sites (Shahid et al. 2012a).

Heavy metal environmental pollution has occurred since ancient times, although their impact became worse during the industrial revolution from increased metal production and from development of new technologies that utilized these metal (Arshad et al. 2008; Nasim and Dhir 2010; Uzu et al. 2010; Vuai and Tokuyama 2011; Pourrut et al. 2011a, 2013; Bai et al. 2011; Tak et al. 2013; Shahid et al. 2013b) (Fig. 1). Unlike organic chemicals, the majority of heavy metals cannot be easily metabolized into less toxic compounds. These metals have long residence times in soils (Radwan et al. 2010; Ahmad and Ashraf 2011; Shahid et al. 2012b), and may continue to exert harmful effects on the environment over long periods

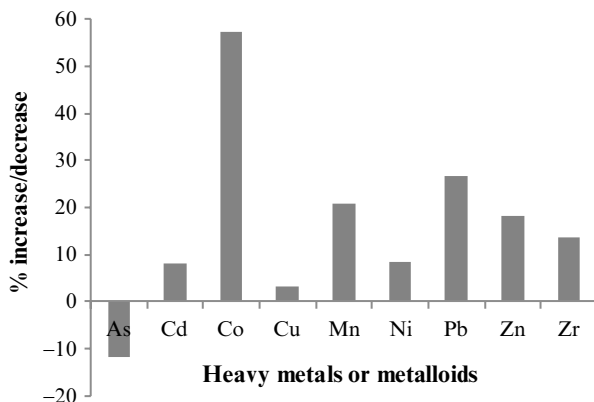


Fig. 1 Percent increase or decrease in annual production of heavy metals and metalloids during the period 2007–2011 [obtained from USGS (2012)]

(Giaccio et al. 2012), thereby representing a potential continuing threat to humans (Kerin and Lin 2010; Uzu et al. 2011a, b; Luo et al. 2012; Zhao et al. 2012; Foucault et al. 2013) and the environment (Schreck et al. 2011; Hunt et al. 2012).

The chemical, biological and physiological effects of heavy metal exposure to plants are of growing concern, because of their potential to accumulate therein and ultimately enter the food chain (Whiteside et al. 2010; Sarma et al. 2011; An et al. 2012; Schreck et al. 2012). The toxic impact of heavy metals on plants have been widely studied (Krzyszowska et al. 2010; Martínez-Fernández et al. 2011; Ahmad et al. 2011a; Evangelou et al. 2012; Hu et al. 2012; Shahid et al. 2013c), and different aspects thereon have been addressed in literature reviews (Pourrut et al. 2011b; Anjum et al. 2012).

Results of previous studies have shown that excessive accumulation of heavy metals in plant tissue can decrease root length, plant biomass, seed germination and chlorophyll biosynthesis (Singh et al. 2010). Inside the cell, heavy metals affect photosynthesis, respiration, mineral nutrition, enzymatic reactions and many other physiological factors (Pourrut et al. 2011b). A rather frequent and common effect of heavy metal toxicity in plants is increased production of reactive oxygen species (ROS). The production of ROS results from the interaction of heavy metals with electron transport activities, particularly in the chloroplast and mitochondrial membranes. The increased production of ROS can disrupt the redox status of cells, resulting in oxidative stress to exposed cells, leading to membrane dismantling, biological macromolecule deterioration, ion leakage, lipid peroxidation and DNA-strand cleavage (He et al. 2011; Carrasco-Gil et al. 2012; Chen et al. 2012). However, the toxic effects of heavy-metal-induced ROS on plant macromolecules vary and depend on the duration of exposure, stage of plant development, concentration of heavy metals tested, intensity of plant stress and the particular organs studied.

To prevent heavy-metal-induced ROS injuries, plants have developed various defense mechanisms by which they can transform ROS into less-toxic products (Tang et al. 2010; Álvarez et al. 2012). These mechanisms include: prohibiting metal entrance into plants, increased root excretion of metals, limiting toxic metal accumulation in sensitive tissue, chelation by organic molecules, metal binding to the cell wall and sequestration in vacuoles. These mechanisms help plants to sustain their cellular redox state and mitigate the damage caused by oxidative stress (Tang et al. 2010). The majority of these defense mechanisms depend on metabolic mediation of natural compounds such as phytochelatins (PCs), reduced glutathione (GSH), carotenoids and tocopherols, and enzymatic antioxidant systems including catalase (CAT and EC 1.11.1.6), superoxide dismutases (SOD and EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), peroxidase (POD, EC 1.11.1.7), guaiacol peroxidase (GPX, EC 1.11.1.7), glutathione reductase (GR, EC 1.6.4.2), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1). The increased levels of these metabolic intermediary compounds and of antioxidant enzymes lead to increased stress tolerance against heavy-metal-induced ROS (He et al. 2011).

Considerable progress has been made in recent years in understanding how different plants respond physiologically to heavy-metal- and metalloid-induced stress. Despite this progress, information is limited on how these plant traits are regulated or are induced. How plants respond physiologically to heavy-metal-induced stress

varies with plant species, metal type and species, and exposure conditions. Additionally, the mechanisms by which heavy metals induce oxidative stress and the different ways in which plants may respond to ROS are not completely elucidated. Therefore, predicting when, or how much heavy-metal-induced ROS production will occur, and how plants will detoxify these ROS are very important steps for improving our ability to assess risks or improve phytoremediation performance. With this in mind, it is our objective in this literature review to summarize key aspects of how plants are affected by heavy-metal-induced ROS production. In particular, we address (1) how plant exposure to heavy metals generates ROS, (2) what the toxic effects of ROS are to plant macromolecules such as DNA, proteins, carbohydrates and lipids, and (3) how plants defend themselves against, and eliminate ROS by enzymatic and non-enzymatic mechanisms.

2 What Are ROS?

“Reactive oxygen species” are generally regarded to exist when the following are present: (1) oxygen-derived free radicals such as hydroxyl (HO^{\bullet}), superoxide anion ($\text{O}_2^{\bullet-}$), peroxy (RO_2^{\bullet}), and alkoxy (RO^{\bullet}) radicals, or (2) oxygen-derived nonradical species such as hydrogen peroxide (H_2O_2), organic hydroperoxide (ROOH) and singlet oxygen ($^1\text{O}_2$) (Corpas et al. 2011; Circu and Aw 2010). Although all of these oxygen-based toxic species are ROS, all ROS are not oxygen radicals. ROS are basically short lived, unstable and chemically very reactive molecules, possessing unpaired valence shell electrons (Wang et al. 2010).

3 ROS Production in Plant Metabolism

3.1 *Natural Production of ROS in Plants*

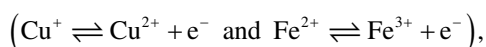
Under aerobic conditions, the generation of ROS is an inevitable aspect of life (Jaspers and Kangasjärvi 2010; Kovacic and Somanathan 2010; Swanson and Gilroy 2010; Wei et al. 2011; Foyer and Noctor 2012). Plant organelles such as mitochondria, chloroplasts and peroxisomes are considered to be major sources of ROS production in plant cells (Karuppanapandian et al. 2011a; del Río 2011; Borisova et al. 2012; Minibayeva et al. 2012; Pucciariello et al. 2012). In sun- or artificial-lighting conditions, peroxisomes and chloroplasts are the main sources of ROS (Foyer and Noctor 2003). However, in darkness, plant mitochondria are considered to be the main site of ROS production (Foyer and Noctor 2003). The main sites of ROS production are the complex I and the complex III of the mitochondrial electron transport chain (Barranco-Medina et al. 2007). It is believed that almost 2% of the O_2 consumed by mitochondria is used to generate H_2O_2 (Becana et al. 2000). In the apoplast, ROS are produced as a consequence of NADPH oxidase activity (Achard et al. 2008; Weyemi and Dupuy 2012; Potocký et al. 2012).

During non-stressed cellular metabolism, O_2 is reduced to H_2O . During this process, ROS such as $O_2^{\cdot-}$, H_2O_2 and OH^{\cdot} are produced as by-products, either by electron transfer or energy transfer reactions (Pucciariello et al. 2012; Borisova et al. 2012). The single electron reduction of O_2 generates the anion superoxide ($O_2^{\cdot-}$). Superoxide is believed to be the precursor of most ROS and acts as a mediator in oxidative chain reactions. This anion is short-lived, which is easily dismutated to H_2O_2 . In contrast to $O_2^{\cdot-}$, H_2O_2 is highly stable and diffusible and is capable of inactivating cell molecules, even at a very low concentration. The main threat imposed by $O_2^{\cdot-}$ and H_2O_2 lies in their ability to generate highly reactive OH^{\cdot} radicals (Møller et al. 2007; Bhatt and Tripathi 2011). In the presence of Fe, H_2O_2 and $O_2^{\cdot-}$ interact in a Haber–Weiss reaction, which produces OH^{\cdot} (Minibayeva et al. 2012). The OH^{\cdot} is considered to be the most reactive ROS, owing to its ability to start radical chain reactions, which are considered to be responsible for producing toxic effects in plants (Mittler et al. 2004; Jones et al. 2011). Under normal conditions, an optimal ROS level is maintained by antioxidant enzymes.

3.2 Heavy-Metal-Induced Production of ROS in Plants

When exposed to heavy metals, plants are known to produce increased quantities of ROS (Table 1). This phenomenon is regarded to be among the earliest of biochemical changes, when plants are subjected to heavy metals stress (Jasinski et al. 2008; Yadav 2010; Grover et al. 2010; Lushchak 2011; Opdenakker et al. 2012). A serious imbalance occurs from the production and elimination of ROS, and this imbalance leads to dramatic physiological challenges to the plant that we call “oxidative stress” (Morina et al. 2010; Kováčik et al. 2010). Metals, such as Cu, Fe, Pb, Cd, Cr, As, Hg, Cr and Zn, all have the ability to induce the formation of ROS (Duquesnoy et al. 2010; Vanhoudt et al. 2010a, b; Márquez-García et al. 2011; Körpe and Aras 2011).

However, the phenomenon of ROS production is different for redox-active and redox-inactive metals (Pourrut et al. 2008; Opdenakker et al. 2012). Redox-active metals such as Fe and Cu catalyze Haber–Weiss/Fenton reactions:



in which H_2O_2 is broken down into OH^{\cdot} at neutral pH (Valko et al. 2006; Sahi and Sharma 2005) (Fig. 2). In contrast, redox-inactive metals, such as Pb, Cd, As, Hg, Ni and Zn inhibit enzymatic activities as a result of their affinity for $-SH$ groups on the enzyme (Mishra et al. 2006; Cuyper et al. 2011; Pourrut et al. 2011b). Redox-inactive metals form covalent bonds with protein sulfhydryl groups because of their electron-sharing affinities. Inactivation of enzymes results from the interaction of heavy metals with proteins, either at the catalytic site or elsewhere. Heavy metals, especially Pb, can also inactivate enzymes by binding to functional groups (COOH) present in proteins (Gupta et al. 2009, 2010). Moreover, displacement of essential cations by heavy metals from specific enzyme binding sites disrupts the ROS balance in cells, and results in ROS overproduction. For example, Zn, which acts as co-factor for many enzymes, can be replaced by heavy metals, causing enzyme

Table 1 Heavy-metal-induced reactive oxygen species (ROS) production in different plant species

Heavy metals	ROS	Plant species	References
Al	OH [•] , H ₂ O ₂ , O ₂ ⁻ NO [•]	<i>Hordeum vulgare</i>	Achary et al. (2012)
		<i>Secale cereale</i>	He et al. (2012)
		<i>Triticum aestivum</i>	
As	NOO [•] , H ₂ O ₂ , O ₂ ⁻	<i>Oryza sativa</i>	Singh et al. (2009)
Cd	H ₂ O ₂ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ H ₂ O ₂ OH [•] , H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ NO [•] , H ₂ O ₂ , O ₂ ⁻ OH [•] , H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ NO [•] H ₂ O ₂ O ₂ ⁻ H ₂ O ₂ O ₂ ⁻ H ₂ O ₂ O ₂ ⁻ H ₂ O ₂ O ₂ ⁻	<i>Arabidopsis thaliana</i>	Martínez-Peñalver et al. (2012)
		<i>Chlorella vulgaris</i>	Piotrowska-Niczyporuk et al. (2012)
		<i>Gracilaria dura</i>	Kumar et al. (2012)
		<i>Brassica juncea</i>	Ahmad et al. (2011b)
		<i>Medicago sativa</i>	Antolín et al. (2010)
		<i>Ipomoea batatas</i>	Kim et al. (2010)
		<i>Alocasia macrorrhiza</i>	Liu et al. (2010a)
		<i>Solanum nigrum</i>	Deng et al. (2010)
		<i>Brassica juncea</i>	Guan et al. (2009)
		<i>Pisum sativum</i>	Rodríguez-Serrano et al. (2009)
		<i>Ceratophyllum demersum</i>	Aravind et al. (2009)
		<i>Triticum aestivum</i>	Singh et al. (2008)
		<i>Arabis paniculata</i>	Qiu et al. (2008)
		<i>Triticum aestivum</i>	Groppa et al. (2008)
		<i>Vicia faba</i>	Lin et al. (2007)
		<i>Mytilus galloprovincialis</i>	Koutsogiannaki et al. (2006)
		<i>Nicotiana tabacum</i>	Olmos et al. (2003)
		<i>Lupinus luteus</i>	Kopyra and Gwóźdź (2003)
		<i>Pisum sativum</i>	Romero-Puertas et al. (2002)
		<i>Oryza sativa</i>	Shah et al. (2001)
Cu	H ₂ O ₂ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ NO [•] , H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻	<i>Pisum sativum</i>	Turchi et al. (2012)
		<i>Arabidopsis thaliana</i>	Martínez-Peñalver et al. (2012)
		<i>Matricaria chamomilla</i>	Kováčik et al. (2010)
		<i>Ipomoea batatas</i>	Kim et al. (2010)
		<i>Medicago sativa</i>	Antolín et al. (2010)
		<i>Lycopersicon lycopersicum</i>	Wang et al. (2010)
		<i>Withania somnifera</i>	Khatun et al. (2008)
		<i>Cucumis sativus</i>	Shi and Zhu (2008)
Mn	H ₂ O ₂ , O ₂ ⁻	<i>Hypnum plumaeforme</i>	Sun et al. (2010)
		<i>Thuidium cymbifolium</i>	
Ni	H ₂ O ₂ , O ₂ ⁻	<i>Brachythecium piligerum</i>	
		<i>Vicia faba</i>	Shahid et al. (2012a, b, c, d)
Pb	H ₂ O ₂ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ OH [•] , H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ , O ₂ ⁻ H ₂ O ₂ H ₂ O ₂ O ₂ ⁻	<i>Chlorella vulgaris</i>	Piotrowska-Niczyporuk et al. (2012)
		<i>Vallisneria spiralis</i>	Wang et al. (2010)
		<i>Spinacia oleracea</i>	Wang et al. (2010)
		<i>Triticum aestivum</i>	Yang et al. (2010)
		<i>Hypnum plumaeforme</i>	Sun et al. (2010)
		<i>Thuidium cymbifolium</i>	
		<i>Brachythecium piligerum</i>	
		<i>Alocasia macrorrhiza</i>	Liu et al. (2010a)
		<i>Medicago sativa</i>	Antolín et al. (2010)
		<i>Sedum alfredii</i>	Liu et al. (2008)
		<i>Vicia faba</i>	Pourrut et al. (2008)
		<i>Elsholtzia argyi</i>	Islam et al. (2008)
		<i>Sedum alfredii</i>	Huang et al. (2008)
		<i>Oryza sativa</i>	Chen et al. (2007)
		<i>Lupinus luteus</i>	Kopyra and Gwóźdź (2003)
Zn	H ₂ O ₂ H ₂ O ₂ O ₂ ⁻	<i>Pisum sativum</i>	Turchi et al. (2012)
		<i>Ipomoea batatas</i>	Kim et al. (2010)
		<i>Mytilus galloprovincialis</i>	Koutsogiannaki et al. (2006)

O₂⁻, superoxide anion; HO[•], hydroxyl; H₂O₂, hydrogen peroxide; NO[•], nitric oxide; NOO[•], nitrogen dioxide

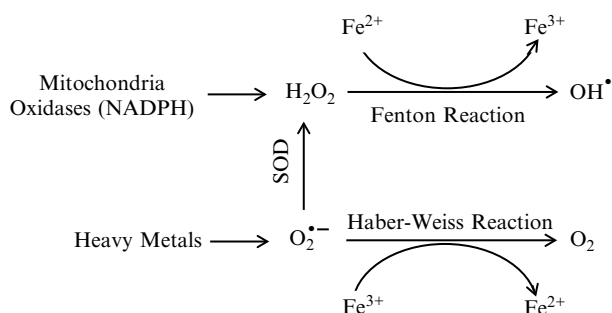


Fig. 2 The Haber–Weiss and Fenton reaction pathways; SOD= Superoxide Dismutase [modified from Kehrer (2000)]

inhibition and oxidative stress. Heavy metals are also capable of depleting GSH inside plant cells (Pourrut et al. 2011b, 2013; Lee et al. 2012). When this happens, heavy metals deplete the major antioxidants that exist within cells, which disrupts the ROS balance. Heavy metals also enhance ROS production via binding and consuming GSH and its derivatives directly, which are required to scavenge any ROS generated (Lee et al. 2003). In addition, plasma-membrane-bound NADPH oxidase is involved in heavy-metal-induced oxidative stress (Sagi and Fluhr 2006; Pourrut et al. 2008, 2013; Weyemi and Dupuy 2012; Potocký et al. 2012). Plasma membrane-bound NADPH oxidases can utilize cytosolic NADPH to generate $O_2^{\bullet-}$, which is quickly dismutated to H_2O_2 by SOD (Pourrut et al. 2008). The ROS formed by the NADPH oxidase exists outside the plasma membrane, where the pH is normally lower than that inside the cell (Sagi and Fluhr 2006). Heavy-metal-induced ROS generation via NADPH oxidase was reported in Cd-treated *Pisum sativum* (Rodríguez-Serrano et al. 2006), Ni-treated *Triticum durum* (Hao et al. 2006) and Pb-treated *Vicia faba* (Pourrut et al. 2008). Moreover, Ca^{2+} and protein kinases have also been reported to have a role in heavy-metal-induced ROS production by activating NADPH oxidase (Yeh et al. 2007; Sahi and Sharma 2005; Pourrut et al. 2013).

4 Roles of ROS in Plant Metabolism

Traditionally ROS were considered to be toxic by-products of aerobic metabolism, but several recent reports clarified the essential roles of ROS in living organisms (Bailly et al. 2008; Rai et al. 2011; Bartoli et al. 2012; Swanson et al. 2011). These essential roles include:

- Plant metabolic defense under stress (Juan et al. 2010; Shin et al. 2011; Rai et al. 2011; Gémes et al. 2011),
- Plant disease resistance (i.e., bacterial and viral) (Jaspers and Kangasjärvi 2010; Shin et al. 2011; Kranner et al. 2010; Rai et al. 2011),

- Plant signal transduction that controls programmed cell death (Pitzschke and Hirt 2006; Blokhina and Fagerstedt 2010; Gill and Tuteja 2010; Rai et al. 2011; Corpas et al. 2011),
- Plant growth regulation (e.g., cell wall loosening) (Kranter et al. 2010; Šírová et al. 2011; Arasimowicz-Jelonek et al. 2011),
- Regulation of photorespiration and photosynthesis (Edreva 2005; Gill and Tuteja 2010),
- Initiating mitogen-activated protein kinase cascades (Jaspers and Kangasjärvi 2010),
- Regulation of root physiology (root hair development, root cell wall loosening and stiffening) (Foreman et al. 2003),
- Regulation of stomatal movement (Yu et al. 2009; Gill and Tuteja 2010),
- Regulation of the cell cycle (Mittler et al. 2004; Gadjev et al. 2008; Gill and Tuteja 2010),
- Fruit ripening and senescence (Karuppanapandian et al. 2011a, b), and
- Alleviation of seed dormancy (Oracz et al. 2009; Kranter et al. 2010; Whitaker et al. 2010; Roach et al. 2010).

The role of H_2O_2 as a signaling molecule, when it intervenes to defend against heavy metal stress has gained considerable attention in recent years. H_2O_2 can mediate the activities of protein kinases, protein phosphatases and transcription factors (Opdenakker et al. 2012). Protein kinases can regulate gene transcription by repressing or activating transcription factors (Pandey and Somssich 2009). Several authors have reported that ROS and protein kinases are activated, in response to heavy metal exposure. Yeh et al. (2007) reported the induction of kinases via ROS production from Cu^{2+} and Cd^{2+} stress. Moreover, cadmium exposure is reported to have induced protein kinase transcripts via the accumulation of ROS in *Zea mays* (Wang et al. 2010) and *Arabidopsis thaliana* (Liu et al. 2010). However, very little is known about the mechanisms and the exact signaling pathways that operate behind these processes in plants that are under heavy metal stress.

5 Toxic Effects of Heavy-Metal-Induced ROS on Macromolecules in Plants

Heavy-metal-induced ROS can elicit widespread damage to plants, examples of which are enzyme inhibition, protein oxidation, lipid peroxidation and DNA and RNA damage (Martínez Domínguez et al. 2009; Cuyper et al. 2011). It has been reported that the indirect effect of heavy metals on plants macromolecules via ROS production is more toxic and rapid than the direct effect (Pourrut et al. 2011b). Reactive oxygen species are involved in the early steps of heavy-metal-induced toxicity to plants, and hence act as initiators of heavy metal toxicity (Shahid et al. 2012c; Martínez-Peñalver et al. 2012).

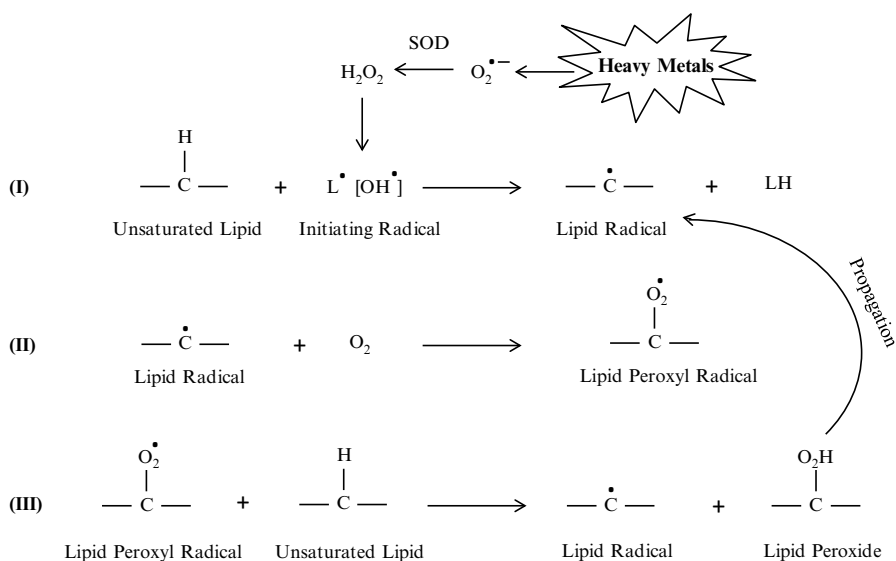


Fig. 3 Depictions of the possible mechanisms by which metals induce lipid peroxidation. The mechanism of heavy-metal-induced lipid peroxidation is initiated most likely via OH^\bullet . The process involves three distinct stages: initiation, progression and termination [modified from Bhattacharjee (2005)]

5.1 Lipid Peroxidation

Lipids are very important cellular components that play vital roles in various biological processes, such as providing energy for cellular metabolism, building cell membranes, and maintaining organelle and cell integrity and composition (Wallis and Browse 2002; Xiao and Chye 2011). Inside the plant, plasma cell membranes are the primary target of heavy metal action (Cuypers et al. 2011). Heavy metals are known to cause lipid peroxidation via ROS production (Fig. 3) (Cuypers et al. 2011; Wahsha et al. 2012; Márquez-García et al. 2012; Chen et al. 2012). Lipid peroxidation causes deterioration of cell membranes, and is one of the most harmful effects induced in plants by heavy-metal exposure (Pourrut et al. 2013). Lipid peroxidation may result from increased lipoxygenase activity, which initiates the formation of oxylipins (Porta and Rocha-Sosa 2002). Lipoxygenase has been reported to play an important role in heavy-metal-induced oxidative stress in *Gracilaria dura*, *Lessonia nigrescens* and *Arabidopsis thaliana* (Smeets et al. 2008; Kumar et al. 2012; Vanhoudt et al. 2011).

The phenomenon of lipid peroxidation is most common in polyunsaturated fatty acids and involves three distinct stages: initiation, progression and termination (Pourrut et al. 2011b; Bhattacharjee 2012). Reactive oxygen species are the most common initiators of lipid peroxidation in living cells. These ROS remove

the hydrogen atom from a methylene group ($-\text{CH}_2-$), thus, giving rise to peroxy radicals (Grover et al. 2010; Singh et al. 2010). The ROS-induced initiation of lipid peroxidation varies with stress condition and cell type. Under normal conditions, lipid peroxidation in green plant tissues is generally initiated by $\text{O}_2^{\cdot-}$, a non-radical electrophilic by-product of light capture in photosystem II (PSII) (Triantaphylidès and Havaux 2009). Heavy metals are known to inhibit PSII, and thus increase $\text{O}_2^{\cdot-}$ production in leaves, which leads to increased lipid peroxidation (Triantaphylidès et al. 2008; Triantaphylidès and Havaux 2009; Farmer and Mueller 2013). In chlorophyll-lacking tissues, lipid peroxidation is started by OH^{\cdot} , a radical produced by Fe- or Cu-catalysed degradation of H_2O_2 (Farmer and Mueller 2013). Although $\text{O}_2^{\cdot-}$ and H_2O_2 are capable of initiating the reactions that are responsible for lipid peroxidation, only OH^{\cdot} is sufficiently reactive, especially in the presence of transition metals such as Cu or Fe (Bhattacharjee 2005; Pourrut et al. 2013). One electron redox cycle results in the formation of peroxy and alkoxy radicals (Karuppanapandian et al. 2011a). The fatty acid radical formed is not very stable. In an aerobic environment, oxygen reacts with the fatty acid, thereby creating another unstable peroxy-fatty acid radical. Once initiated, ROO^{\cdot} groups are capable to continue the peroxidation chain reaction by receiving a hydrogen atom from neighbouring polyunsaturated fatty acids (Bhattacharjee 2005; Karuppanapandian et al. 2011a). The resulting lipid hydroperoxide is a highly unstable molecule and decays into several reactive species such as lipid epoxides, aldehydes (malonyldialdehyde), lipid alkoxy radicals, alkanes and alcohols (Bhattacharjee 2005). The cycle continues from the presence of fatty acid side chains that are in close proximity to plant membranes, which facilitates autocatalytic propagation of lipid peroxidation.

Generally lipid peroxidation causes: (1) increased membrane leakiness to substances that do not normally cross membranes, other than via specific channels, (2) decreased membrane fluidity, which makes it easier for phospholipids to be exchanged between the two halves of the bilayer, and (3) damage to membrane proteins that inactivate receptors, enzymes, and ion channels. Several studies revealed toxic effects from lipid peroxidation in plants (Yamauchi and Sugimoto 2010; Farmer and Mueller 2013). Some recent studies reported that heavy metal toxicity to different physiological processes occurs via ROS-induced lipid peroxidation (Shahid et al. 2013d). The by-products of lipid peroxidation also strongly affect photosynthetic reactions. For example, acrolein, linolenic acid-13-ketotriene and 12-oxo-phytodienoic acid are well known to induce toxic effects on PSII (Alméras et al. 2003). Exogenous acrolein is reported to deplete chloroplast glutathione pools (Mano 2012). Lipid peroxidation also causes covalent modification of plant proteins due to the binding of electrophilic lipid fragments with proteins (Farmer and Mueller 2013). This covalent binding occurs when nucleophilic atoms (e.g., S or N) bind to the β -carbon of α,β -unsaturated carbonyl groups. Nowadays, increased attention is being given to the damaging effects of lipid peroxidation products, which can be monitored by using of transgenic approaches (Mano 2012).

5.2 DNA Damage

Heavy-metal-induced genotoxicity in plant cells is a complex phenomenon, and the mechanisms behind this process are not yet well understood (Aina et al. 2004; Tuteja et al. 2009; Cuyper et al. 2011; Zhu et al. 2011; Shen et al. 2012). According to some authors, heavy-metal-induced DNA damage is not direct but occurs indirectly through ROS production (Gichner et al. 2006; Gupta and Sarin 2009; Barbosa et al. 2010; Hirata et al. 2010, 2011). Heavy-metal-induced DNA damage has been reported in several plants, examples of which are, *Trifolium repens* (Aina et al. 2004), *Cannabis sativa* (Aina et al. 2004), *Allium cepa* (Barbosa et al. 2010), *Vicia faba* (Marcato-Romain et al. 2009a; Pourrut et al. 2011c), *Boletus edulis* (Collin-Hansen et al. 2005), and *Nicotiana tabacum* and *Solanum tuberosum* (Gichner et al. 2006).

Among ROS, OH[•] is the most reactive entity in damaging all components of the DNA molecule (Jones et al. 2011). Reactive oxygen species interactions with DNA results in: damage to cross-links, base deletions, base modifications, strand breaks and damage to pyrimidine dimers (Tuteja et al. 2001; Gastaldo et al. 2008). Among these affected DNA sites, base deletion is the most frequent DNA damage induced by either heavy metals, ionizing radiation or ultra violet radiation (Gastaldo et al. 2008). DNA has four different potential sites to which metals may bind, i.e., the ribose hydroxyls, the negatively charged phosphate oxygen atoms, the exocyclic base keto groups and the base ring nitrogens (Oliveira et al. 2008). Most transition metal ions interact in a complex way with DNA: more than two different sites are generally involved. Heavy metals generally bind directly to the bases, with the N7 atom of purines or N3 of pyrimidines and indirectly to the phosphate groups (Anastassopoulou 2003). In vitro studies indicated that heavy metals like Cd, Cr, Cu, Hg, Pb and Zn interact with DNA, particularly at sulfhydryl groups and the phosphate backbone (Sheng et al. 2008). Moreover, heavy metals may alter gene expression (Rossman 2000) and they appear to interact with Zn-fingers, which bind tetrahedrally to cysteine (Cys) thiolates and/or histidine imidazole groups to maintain the DNA three-dimensional structure (Witkiewicz-Kucharczyk and Bal 2006). DNA damage can occur either from replication errors, induction of signal transduction pathways, induction of transcription, cell membrane destruction and/or genomic instability (Cooke et al. 2003). In plants and other living organisms, damage inflicted on DNA and repair mechanisms generally occur concomitantly, making these processes both complex and difficult to independently assess (Gastaldo et al. 2008).

When ROS interact with DNA, oxidized bases are frequently generated (Hirano and Tamae 2010). Among the different forms of oxidative DNA damage, effects with 8-oxoguanine has been most extensively investigated (Hirano and Tamae 2010), and is also an event that may lead to neoplastic transformation (Bal and Kasprzak 2002). Using a plasmid-relaxation assay, Yang et al. (1999) demonstrated that Pb and Cd promoted DNA strand-breakage and formed 8-hydroxydeoxyguanosine (8-OHdG) adducts in DNA. Recently, Hirata et al. (2011) showed As- and Cr-induced translesion DNA synthesis due to their increased affinity for DNA containing 8-OHdG.

Heavy-metal-induced damage to DNA may also result in the production of micronuclei, which produce chromosome breaks or mitotic anomalies that require passage through mitosis to be recognisable (Marcato-Romain et al. 2009b). According to Johnson (1998), heavy metals are capable of interfering with the spindle apparatus of dividing cells to produce DNA damage. Cenkci et al. (2009) described Pb-induced genotoxicity, using a random amplified polymorphic DNA (RAPD) profile, in *Brassica rapa* exposed to 0.5 to 5 mM concentrations of lead nitrate. Radić et al. (2011) demonstrated damage to DNA (estimated by tail extent moment) in *Lemna minuta* exposed to heavy metals from industrial wastewater. Recently, Shahid et al. (2011) reported the Pb-induced production of micronuclei in *Vicia faba* root tips via ROS production. More recently, Pourrut et al. (2011b) demonstrated a close link between oxidative stress induced by Pb, DNA strand breaks and micronuclei formation in *Vicia faba* root tips.

5.3 Protein Damage

Heavy metals may also cause toxic effects in the structure of plant proteins (Tan et al. 2010; Luque-Garcia et al. 2011). Protein synthesis is the primary target of ROS damage in plants (Nishiyama et al. 2011). This heavy-metal-induced change in protein quantity or quality can occur via several mechanisms, e.g., binding of the metal ions to free thiols and other functional groups of proteins, replacement of Zn and other essential metal ions by free heavy metal ions in metal-dependent proteins, etc. Whatever the location of heavy metal-induced ROS, they generally interact with proteins that contain sulfur-containing amino acids and thiol groups. Proteins are more susceptible to heavy metal ions during the process of folding, than are proteins that have already reached their native state (Sharma et al. 2008).

Heavy-metal-induced ROS also cause a quantitative reduction in total protein content of cells (Mishra et al. ; Garcia et al. 2006). This quantitative decrease in total protein content results from various heavy metals effects: they modify gene expression (Kovalchuk et al. 2005), increase ribonuclease activity (Gopal and Rizvi 2008), consume amino acids to scavenge ROS (Gupta and Sinha 2009), and reduce free amino acid content (Gupta et al. 2009) that is linked to alteration in nitrogen metabolism (Chatterjee et al. 2004). Heavy metal ions form complexes with proteins by binding with $-\text{COOH}$, $-\text{NH}_2$ and $-\text{SH}$ groups (Tan et al. 2010). As a result, these modified biological molecules cannot function properly as a result of their structural modification, and this produces cell malfunction. When heavy metals bind to these active groups of proteins, they inactivate different enzyme systems, or alter protein structure, which is related to the catalytic properties of enzymes. Reactive oxygen species do oxidize the following protein amino acid side groups: Cys, Met, His, Arg, Lys, Pro, Tyr and Trp. Cadmium treatment raised the carbonylation level from 4 to 5.6 nmol/mg protein in *Pisum sativum* plants (Romero-Puertas et al. 2002). Most of these reactions are irreversible, although in the specific case of thiol-group oxidation, enzyme-catalyzed re-reduction is possible (Rouhier et al. 2006).

Recent findings suggest that protein oxidation events are most likely to occur in proteins that are extremely close to the site of ROS production. Certain metal ion co-factors, such as Fe-S, are particularly susceptible to oxidation. Heavy metal exposure to plants not only causes a quantitative change to protein content, but also may alter the qualitative composition of cell proteins. The protein composition of root cells in *V. faba* seedlings was altered when exposed to Pb (Beltagi 2005), and this can result from the modification in transcriptome profile of numerous enzymes such as: cysteine proteinase, isocitrate lyase, arginine decarboxylase and serine hydroxymethyltransferase (Kovalchuk et al. 2005).

Heavy metals also may produce indirect effects on protein functioning that curtails protein synthesis or inhibits protein functioning (Pena et al. 2008). For example, the plant proteolysis system helps to regulate protein processing and intracellular protein levels, and removes abnormal or damaged proteins from the cell (Buchanan et al. 2000). The proteolytic system is mainly localized inside certain organelles, e.g., cytoplasm and the nucleus (Rawlings 2004). Cadmium has been reported to cause oxidation of the proteasome in *Zea mays* (Pena et al. 2007) and *Helianthus annuus* plants (Pena et al. 2006). This enhancement of the proteasome activity prevents accumulation of oxidatively damaged proteins in the cell (Pena et al. 2007).

5.4 Damage to Plant Carbohydrates

Carbohydrates are ubiquitous energy sources, and are key macromolecules for their role in plant metabolism and structure (Guan-fu 2011; Dong et al. 2011). Carbohydrates are the major products of photosynthesis and act as transport molecules in plant growth, development and storage (Couée et al. 2006). They are involved in response mechanisms to different stressors, osmotic adjustment, and nutrient and metabolic signaling molecules (Hummel et al. 2009). They also help to maintain plasma membrane integrity (Guan-fu 2011), feed the NADPH-producing metabolic pathways involved in ROS scavenging, and interact with plant hormone signaling through molecules such as the auxins and cytokinins (Rolland et al. 2002), gibberellin, abscisic acid and ethylene (Price et al. 2004). Heavy metals are known to affect plant sugar content through ROS-induced oxidative stress. Interaction between soluble sugar content and ROS cause pollen abortion in *Triticum aestivum* (Lehner et al. 2008) or decreased pollen viability in *Oryza sativa* (Guan-fu 2011), which might be due to the interplay between programmed cell death and ROS. Any expression of sugar transporter genes that are induced by heavy metal stress may reduce the oxidant damage caused by overproduction of ROS (Nguyen et al. 2010). Glucose is reported to enhance cellular defences against cytotoxicity of H₂O₂ in plants, and enhances plantlet survival (Averill-Bates and Przybytkowski 1994). Under intense oxidative stress conditions, ROS affects the structure of carbohydrates (Zadák et al. 2009). When thus affected, plant defense mechanisms are weakened and plant macromolecules (including glucose) become vulnerable to heavy metal toxicity.

5.5 Interference with Signalling

Heavy metals interfere with cell signalling via mechanisms that are poorly understood. Effects of heavy metals on cell signalling may be direct as a result of the interaction of metals with proteins, or indirect from the formation of metal-induced ROS. It has been proposed that heavy-metal-induced dysregulation of signalling events play a key role in the response of heavy metal toxicity as well as in damage development. Metals affect the gene expression, transcription and activation of numerous signalling proteins, including growth factor receptors, G-proteins and tyrosine kinases (Harris and Shi 2003). In plants, several studies have shown that heavy metals (Cu, Zn, Pb and Cd) intervene with mitogen kinase signalling cascades. Mitogen-activated protein kinase (MAPK) pathways incorporate various signalling stimuli, and specific elements are also activated by ROS (Zhang and Klessig 2001). These MAPKs are rapidly activated in *Medicago sativa* by an excess of Cu (Jonak et al. 2004). However, Cd exposure activates MAPKs in *Medicago sativa* after a considerable delay (Jonak et al. 2004). The titer of jasmonic acid, salicylic acid and ethylene increases in plants after exposure to heavy metals (Pál et al. 2005), which then enhances H₂O₂ generation (Zawoznik et al. 2007) and interferes with cell signalling. Romero-Puertas et al. (2007) explained how the redox component scheme works, and explained how signalling molecules positively or negatively adjust the expression of antioxidant genes during long-term Cd stress in *Pisum sativum*.

6 Plant Heavy-Metal Tolerance Mechanisms

To survive, plants have to constantly cope with stress. Certain plants (especially heavy metal hyperaccumulator plants) operate well even under extreme ROS production situations that are caused by heavy metal toxicity. In fact, plants have evolved an array of defense mechanisms to combat oxidative damage, for the purpose of restricting cell injury and tissue dysfunction (Shulaev et al. 2008; Benekos et al. 2010; Ruan et al. 2011). Such defense mechanisms act separately or simultaneously in plants to scavenge any ROS over-production. However, what specific plant defense mechanism are active, and the efficiency of it, depends on the plant species, plant maturity, type of metal involved, and the level and duration of exposure.

Generally, stress-tolerant plants better defend themselves against ROS than do stress-susceptible species (Liu and Pang 2010). Hyperaccumulator plants are efficient at detoxifying and sequestering heavy metals, which enable them to accumulate high metal levels in their shoot tissues, without suffering phytotoxic effects (Rascio and Navari-Izzo 2011). Such preferential heavy metal detoxification/sequestration does occur in specific plant structures, such as the epidermis (Freeman et al. 2006), trichomes (Küpper et al. 2000) and even the cuticle (Robinson et al. 2003), where they cause toxicity to the photosynthetic apparatus, if not detoxified.

6.1 Primary Heavy-Metal Tolerance Mechanisms

Heavy metals mainly enter plants from soil through the roots (Uzu et al. 2009; Tang et al. 2010). Heavy metals, especially Pb, are adsorbed onto the root surface before uptake and become bound to carboxyl groups of mucilage uronic acid or to the polysaccharides of the rhizoderm cell surface (Seregin et al. 2004; Pourrut et al. 2011b). Such binding of heavy metals to exchange sites at the root surface is a commonly employed plant strategy to limit heavy metal absorption into root cells; the entrapment occurs in the apoplast by binding the metals to exuded organic acids or anionic groups of cell walls (Jiang and Liu 2010). In response to heavy metal toxicity, root thickness can increase, and thereby increase the amount of metal adsorbed onto the root surface; when this occurs, the consequence is to reduce metal penetration into roots (Krzyszowska et al. 2009, 2010). Probst et al. (2009) observed increased cell wall thickness of *Vicia faba* as an ultrastructural alteration caused by a high metal level. Liu et al. (2004) and Andrade et al. (2004) reported similar increases in cell wall thickness, respectively, in shoots of *Vicia faba* that were exposed to Cu or Cd, and in marine macroalgae exposed to Cu. Such increases are believed to be associated with enhanced peroxidase activity (Liu et al. 2004; Probst et al. 2009). This enzyme catalyzes lignin synthesis (Arduini et al. 1995) and is generally produced in higher plants exposed to heavy metals (Prasad 1996). Probst et al. (2009) observed high amounts of electron-dense particles of metals (Pb and Zn) on the surface, and within the cell walls of *Vicia faba* roots. Similar Pb deposits were shown to exist along plasma membranes of *Sesbania* root cells by Sahi and Sharma (2005). Krzyszowska et al. (2009) reported reduced penetration of Pb into the plasma membrane in *Funaria hygrometrica* from increased cell wall thickness, as a result of Pb binding with JIM5-P, within the cell wall. However, Pb bound to JIM5-P can be remobilized by endocytosis (Krzyszowska et al. 2010). It has been reported in several studies that Pb is adsorbed onto roots in many plant species: *Vigna unguiculata* (Kopittke et al. 2007), *Brassica juncea* (Meyers et al. 2008), *Festuca rubra* (Ginn et al. 2008), *Lactuca sativa* (Uzu et al. 2009) and *Funaria hygrometrica* (Krzyszowska et al. 2010). The degree of adsorption of metals onto plant root surface varies with the physico-chemical properties of rhizosphere soil, and plant and metal type (Saifullah et al. 2009; Pourrut et al. 2011b). The adsorption of metals onto root surfaces reduces their entrance into plants, which is considered to be beneficial in the case of vegetables (Pourrut et al. 2011b).

Another defense mechanism plants adopt is to reduce the translocation of heavy metals to aerial plant parts. Most of the heavy metals absorbed by plants are sequestered in plant root cells. In root cells, toxic metals are detoxified by complexation with organic acids, amino acids or sequestered into vacuoles (Rascio and Navari-Izzo 2011; Pourrut et al. 2011b). Such complexation restricts the transfer of heavy metals towards aerial plant parts, thus protecting leaf tissues, and particularly the metabolically active photosynthetic cells from heavy metal damage (Rascio and Navari-Izzo 2011). Increased sequestration of heavy metals in root cells is achieved

by several mechanisms: they precipitate as insoluble salts in intercellular spaces (Meyers et al. 2008), they are immobilized by negatively charged pectins within the cell wall (Arias et al. 2010), they accumulate in plasma membranes (Jiang and Liu 2010), or are sequestered in the vacuoles of rhizodermal and cortical cells (Kopittke et al. 2007). Many researchers have reported that >90% of heavy metals present accumulate in plant root cells of many plant species. Examples are: *Vigna unguiculata* (Kopittke et al. 2007), *Pisum sativum*, *Phaseolus vulgaris* and *Vicia faba* (Pourrut et al. 2011a), *Arabidopsis thaliana* (Vanhoudt et al. 2010a) *Avicennia marina* (Yan and Lo 2011), *Sedum alfredii* (Gupta et al. 2010), *Allium sativum* (Jiang and Liu 2010), *Lolium perenne* (Jia et al. 2011), *Oryza sativa* (Hu et al. 2011), *Erica andevalensis* (Mingorance et al. 2012) and *Chrysopogon zizanioides* (Danh et al. 2011). The phenomenon of increased amounts of metals being restricted to accumulating in roots is more common to Pb than to other heavy metals.

6.2 Secondary Heavy-Metal Tolerance Mechanisms

When plants take up high levels of heavy metals, toxicity is prevented only if the plants have a strong sink adequate for storing the toxic metals (Wojas et al. 2010; Hassan and Aarts 2011). By having such sinks, plants can evade the toxic effects of these metals. Vacuolar sequestration is an important feature that maintains plant metal homeostasis, and detoxifies heavy metals (Maestri et al. 2010). The hyperaccumulator plants have the ability to limit negative effects of metals by sequestering and/or binding them to molecules or plant structures. Heavy metals are detoxified in aerial parts of hyperaccumulators plants as a result of ligand binding or entrapment by vacuoles (Rascio and Navari-Izzo 2011). Vacuolar transporters partly fulfil this role, by contributing to the partitioning of metals into the vacuole (Martinoia et al. 2007).

The vacuole is the final destination for practically all toxic substances. There are several pathways by which metals are sequestered vacuoles. Genomic sequencing analysis has identified various families of transporters that are involved in heavy metal homeostasis in plants (Klatte et al. 2009; Chaffai and Koyama 2011). These transporter families include ATP-binding cassettes (ABC), heavy metal ATPases (HMAs), Zrt/Irt-like protein (ZIP), cation exchangers (CAXs), natural resistance-associated macrophage (NRAMP) and cation diffusion facilitators (CDF) (Grotz and Gueriot 2006; Hall and Williams 2003). Among these, CDF ABC and NRAMP have been identified as being critical for heavy metal tolerance (Hanikenne et al. 2005; Chaffai and Koyama 2011).

Metallothioneins (MTs) and phytochelatins are the best characterized and important metal-binding ligands in plant cells (Rea 2012). Phytochelatins are small, heavy-metal-binding polypeptides that have the general structure of (γ -Glu-Cys) n Gly ($n=2-11$). Phytochelatins belong to different classes of cysteine-rich heavy metal-binding protein molecules. Heavy metals are capable of stimulating the production of PCs, and activating the enzyme phytochelatin synthase (PCS) (Vadas and Ahner 2009; Jiang and Liu 2010). The synthesis of PCs is catalyzed

non-translationally by PCS, which is activated by metal ions such as Cd, Pb, Zn, and Cu (Andrade et al. 2010; Ogawa et al. 2011). In plants, these natural chelators bind and transport heavy metals to cell vacuoles (Israr et al. 2011). The transport of the metal-PC complex to vacuoles is thought to be facilitated by ABC transporters (Prévéral et al. 2009; Park et al. 2012), which for *Oryza sativa* seedlings, are encoded by OsPDR5/ABCG43 (Oda et al. 2011). PCs bind and transport heavy metals by forming mercaptide bonds with them (Verbruggen et al. 2009; Semane et al. 2010). Generally, PCs bind metals in the cytosol, and the resulting PC-metal complex is sequestered in vacuoles (Ogawa et al. 2011), thereby reducing the concentration of free metal ions in the cytosol. In this way, these natural ligands inhibit ROS production that results from heavy metal interactions with the delicate redox system. In in-vivo studies, Yadav (2010) reported that PCs were involved in the cellular detoxification and accumulation of heavy metals as a result of their ability to form stable metal-PC complexes. Gisbert et al. (2003) reported that the induction and over-expression of a *Triticum aestivum* gene encoding phytochelatin synthase (TaPCS1) significantly increased uptake and tolerance of *Nicotiana glauca* to Pb and Cd.

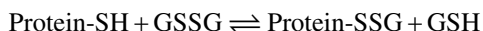
Glutathione (GSH; γ -glutamylcysteine-glycine), a sulfur containing tri-peptide, is among the most important and critical of the low molecular weight biological thiols. Glutathione protects plants from heavy metal toxicity by quenching metal-induced ROS (Vanhoudt et al. 2010a; Seth 2012; Noctor et al. 2012). Glutathione reacts nonenzymatically with a series of ROS by forming thiyl radicals (Halliwell and Gutteridge 1999). Thiyl radicals may generate $O_2^{\cdot-}$, which can be neutralized by SOD/CAT enzymes. It is worth noting that GSH also reacts with the lipid peroxidation metabolite 4-hydroxy-2-nonenal (Wonisch et al. 1997), and plays a role in the initial resistance against malondialdehyde, another highly toxic lipid peroxidation product (Turton et al. 1997).

Moreover, it is a substrate for PC biosynthesis, and certain related proteins play a key role in detoxifying heavy metals (Huang and Wang 2010; Ogawa et al. 2011). It is noteworthy that metals do not directly activate PCS activity, but rather, a GSH-metal complex is formed, (i.e., in which the metal binds to a thiol group), which activates PCS (Na and Salt 2010). Glutathione synthesis is catalyzed by two ATP-dependent enzymes, γ -glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). Heavy metal exposure can induce different GSH genes, such as glutathione synthetase, glutamyl cysteine synthetase, glutathione peroxidase and glutathione reductase. A deficiency of GSH affects defense gene expression and the hypersensitive response in plants (Dubreuil-Maurizi et al. 2011). Glutathione is reported to enhance proline accumulation in heavy-metal-stressed plants, a role that is correlated with reduced damage to membranes and proteins (Liu et al. 2009). Generally, PCs and GSH are simultaneously stimulated in plants to detoxify heavy metals. However, Gupta et al. (2010) reported the induction of GSH alone for detoxification of heavy metals in *Sedum alfredii*. The enhanced production of GSH does not always increase plant tolerance or detoxify heavy metals to reduce plant stress (Xiang et al. 2001). Therefore, GSH alone may not be adequate to resist heavy-metal stress in plants (Noctor et al. 1998; Yadav 2010).

Glutathione also plays an important indirect role in detoxifying heavy metals via activating the PCS enzyme. Once sufficient GSH levels are achieved during heavy metal stress, PCS become active and catalyzes the formation of PC–metal complexes (Yadav 2010). PCS are activated when a heavy metal and two GSH molecules form a thiolate complex (Cd–GS₂ or Zn–GS₂). Activation of PCS also results in the transfer of one γ -Glu-Cys moiety to a free GSH molecule or to a previously synthesized PC (Singla-Pareek et al. 2006). Depletion of GSH may result from its consumption for PCs synthesis (Mishra et al. 2006), or from direct binding with heavy metal ions (Andra et al. 2009a, b).

6.3 Glutathionylation

The thiol group of the amino acid cysteine is extremely vulnerable to ROS (oxidative damage), due to its high sensitivity to oxidation. To protect proteins from oxidation, plant cells have developed a tolerance mechanism, glutathionylation, which results in a reversible posttranslational modification of protein thiols (Michelet et al. 2006; Zaffagnini et al. 2012a). During glutathionylation, the protein thiols are oxidized to various reversible products, such as S-glutathionylation, sulfenic or sulfinic acids, and intra- or inter-protein disulfide bonds (Li and Zachgo 2009). The reaction mechanism of glutathionylation involves an exchange of a thiol/disulfide between GSSG and a protein thiol as following:



Several proteomic studies have demonstrated the glutathionylation of a number of chloroplast proteins under oxidative stress conditions (Ito et al. 2003; Zaffagnini et al. 2007, 2012a, b). The glutathionylation reaction is generally supported by ROS such as H₂O₂ under stress conditions (Zaffagnini et al. 2012b). In the absence of a glutathionylation reaction, the thiol group of cysteine could be oxidized to irreversible forms, i.e., sulfates and sulfonates (Poole et al. 2004). In this way, the reaction of GSH with thiol groups of cysteine (glutathionylation) protects proteins from possible damage by ROS on redox signaling, although it has yet to be completely elucidated and is currently under extensive investigation (Zaffagnini et al. 2012a).

A number of redoxactive enzymes are known to intervene in the glutathionylation process. Examples, on which we elaborate below, are the peroxiredoxins (PRDXs) (Dietz 2003; Zaffagnini et al. 2012a), glutaredoxins (GRXs) (Xing et al. 2006; Meyers et al. 2008), thioredoxins (TRXs) (Buchanan and Balmer 2005; Zaffagnini et al. 2012a), and protein disulfide isomerases (Alergand et al. 2006). These redoxactive enzymes, together with a various redox-active target proteins defend proteins from irreversible oxidation especially under oxidative stress conditions (Ströher and Dietz 2006; Meyers et al. 2008; Zaffagnini et al. 2012a).

Peroxiredoxin (PRDXs) comprises a family of thiol-based peroxidases found in organisms ranging from bacteria to mammals (Abbas et al. 2008; Bhatt and Tripathi 2011; Anjum et al. 2012; Djuika et al. 2013). Though the roles of PRDXs have not yet been completely elucidated, their role in heavy-metal-induced ROS detoxification is evident (Matamoros et al. 2010; Abbas et al. 2013). The proteomic analysis of maize roots (Requejo up-regulation of PRDXs under heavy metal stress. These enzymes usually catalyze the reduction of H_2O_2 and other hydroperoxides (ROOH) with help from reduced thioredoxins, to yield thioredoxin disulfide, water, and the corresponding alcohol (Dietz 2011; Deponte 2013; Djuika et al. 2013; Randall et al. 2013). Bhatt and Tripathi (2011) described the reaction mechanism of PRDXs-induced decomposition of $O_2^{\cdot-}$ to H_2O . They summarized the entire process in three steps: peroxidation, redox dehydration and reduction as reported by Aran et al. (2009). The reaction starts as a nucleophilic attack of the protein thiol on the peroxide, resulting in the release of an alcohol and concomitant oxidation to a sulfenic acid (RSOH), which starts the catalytic cycle (Ellis and Poole 1997). The thiol group of Cys attacks RSOH, resulting in the release of H_2O and formation of a disulfide bridge. The catalytic cycle is stopped by a complementary reduction system, which results in catalytically active PRDXs (Aran et al. 2009; Bhatt and Tripathi 2011). Peroxiredoxin with CAT and other peroxidases are reported to take part in signal transduction by controlling the intracellular H_2O_2 concentration (Randall et al. 2013; Poynton and Hampton 2013). In plants, PRDXs have four subgroups (1-Cys PRDX, 2-Cys PRDX, PRDX II and PRDX Q) that are based on the number and position of the conserved cysteine residues, genome-wide analysis of plants and their subunit composition (Rouhier et al. 2001; Rouhier and Jacquot 2002; Poynton and Hampton 2013).

Thioredoxin (TRXs) is a family of antioxidant redox proteins (12.4 kDa) that facilitate the reduction of other proteins through the exchange of thiol/disulfide (Lemaire et al. 2003). For example, thioredoxins act as hydrogen donors for thioredoxin peroxidases or peroxiredoxin, which are involved in the removal of H_2O_2 (Verdoucq et al. 1999; Behm and Jacquot 2000). The reaction mechanism involves the reduction of the oxidized disulfide form of thioredoxin by NADPH and thioredoxin reductase (TRR). Depending on the primary sequence and sub-cellular localization, plants have six subgroups/types (TRXs f, m, x, y, h, and o). These subgroups have different sub-cellular compartmentalization and function. Thioredoxin-x, -y, -z, and NTRc are reported to act as electron donors to various antioxidant enzymes such as the glutathione peroxidases, methionine sulfoxide reductases and peroxiredoxins (Tarrago et al. 2009; Chibani et al. 2010).

However, it is not always evident that ROS detoxification by antioxidant enzymes requires electrons from the glutaredoxin or thioredoxin systems (Culotta et al. 2006; Benabdellah et al. 2009). It is reported that in GSH deficient cells, TRXs are over-produced to compensate for GSH shortage (Pócsi et al. 2004). Examination of the redox state of TRXs and GRXs in mutant plants showed that TRXs are independent of the GSH/GRX system (Trotter and Grant 2003). Still the interaction of TRXs,

GRXs and GSH in redox-dependent regulation, based on disulfide/dithiol exchange reactions under stress conditions (overproduction of ROS), is not well established in plants.

Glutaredoxins (GRXs) are oxidoreductases that catalyze the reversible reduction of disulfide bonds and participate in antioxidant defence by reducing various enzymes such as peroxiredoxins, dehydroascorbate, and methionine sulfoxide reductase (Buchanan and Balmer 2005; Li and Zachgo 2009). Glutaredoxins are oxidized by substrates, and reduced non-enzymatically by GSH. In the dithiol mechanism, electrons are transferred from NADPH to GR, then to GSH, and from there to GRXs. Finally, GRXs reduce target proteins by dithiol-disulfide exchange reactions in a manner similar to TRXs. The plant glutaredoxin family contains more than 30 members that are localized in different cell compartments (Couturier et al. 2009; Zaffagnini et al. 2012b). Almost thirty different GRXs isoforms have been identified in *A. thaliana*. They are subgrouped in six classes based on their redox-active center (Xing et al. 2006). Each class contains a variant of the active site motif and peculiar functional properties (Rouhier et al. 2006). GPXs appears to be involved in detoxifying H₂O₂ (Foyer and Noctor 2005, 2009) as well as lipid and phospholipid hydroperoxides (Avery and Avery 2001). GRXs also participate to reduce the oxidized cysteines, providing evidence of GRXs role in oxidative stress signaling (Michelet et al. 2006).

6.4 Nitrogen Metabolism

Nitrogen metabolism plays an important role in plant responses to heavy metal toxicity (Lea and Azevedo 2007; Andrade et al. 2010). Various nitrogenous metabolites, such as polyamines, amino acids and amino acid-derived molecules can bind to and scavenge heavy-metal-induced ROS (Kovac et al. 2009; Radić et al. 2010). When plants are exposed to high heavy metals levels, it is reported that some plant amino acids (e.g., proline or histidine), scavenge ROS (Sharma and Dietz 2006; Fariduddin et al. 2009).

Huang and Wang (2010) suggested that free prolines help protect certain plant enzymes, osmoregulation and help to stabilize the sub-cellular components and structures. Proline has been reported to accumulate in plants under heavy metal stress conditions, an indication that its increased presence provides a protective or a regulatory role (Sharma and Dietz 2006). Metal-tolerant plants contain higher constitutive proline levels, even in the absence of excess metal ions, than do non-tolerant plants (Sharma and Dietz 2006; Huang and Wang 2010). Increased levels of proline correlate with enhanced metal tolerance in plants, and as a result, some researchers believe it to act as an antioxidant in metal-stressed cells (Gupta and Sarin 2009; Huang and Wang 2010). One of the proposed roles of proline is to reduce free radical levels that are generated from toxicity events. In this regard, proline acts in a manner that is similar to GSH, ascorbic acid or tocopherol. Heavy metals interfere with N metabolism to cause toxicity that alters the composition of amino acid in plants (Callahan et al. 2007).

6.5 Antioxidant Enzymes

One of the most efficient mechanisms that plants use to protect themselves is to detoxify any free radicals that are present. Such detoxification prevents cell injury and tissue dysfunction and is accomplished in plant cells via activation of antioxidants enzymes such as SOD, CAT, POD, APX, GR, DHAR and MDHAR (Table 2, Fig. 4) (Lomonte et al. 2010; Mou et al. 2011; Vanhoudt et al. 2011; Lyubenova and Schröder 2011; Cestone et al. 2012; Opdenakker et al. 2012; Shahid et al. 2013d). Previous results have shown that high levels of antioxidant enzymes can increase stress tolerance to heavy-metal-induced stress conditions. Many researchers have also reported that antioxidant enzymes are activated in different plant species to scavenge the ROS that are produced by heavy metal toxicity (Gonnelli et al. 2001; Kim et al. 2010; Kafel et al. 2010; Martínez Domínguez et al. 2010; He et al. 2011).

Plant species display different levels of tolerance to heavy metal exposure (Shahid et al. 2012d), and the enzymes in these plants display varying behavior when under heavy metal stress. Most of these antioxidative enzymes are electron donors and react with free radicals to form innocuous end products, such as water. The process involves the binding of these ROS to active enzyme sites, and then conversion to non-toxic and inactive products. Among these enzymes, SOD is a key one for defending plants against ROS. The catalytic properties of SOD were first detected by McCord and Fridovich (1969). SOD is responsible for dismutation of the two superoxide radicals to H_2O_2 and O_2 . In this way, SOD maintains $O_2^{\cdot-}$ at a steady state level (Gao et al. 2010; Deng et al. 2010; Andrade et al. 2010; Cestone et al. 2012). An increase in SOD activity could be either direct through the action of heavy metal ions on SOD, or indirect through an increase in $O_2^{\cdot-}$ levels (Chongpraditnun et al. 1992; Shahid et al. 2013d). When SOD appears, it generally does so in response to the production of heavy-metal-induced H_2O_2 , which can form lipid peroxides by direct or indirect action by lipoxygenase-mediated lipid peroxidation (Deng et al. 2010). An increase in SOD activity may result from enhanced formation of $O_2^{\cdot-}$ or from de novo synthesis of enzyme proteins (Verma and Dubey 2003; Yılmaz and Parlak 2011). Catalase is generally present in mitochondria and peroxisomes, where it decomposes H_2O_2 to H_2O and O_2 (Hermes-Lima 2005; Tang et al. 2010; Shahid et al. 2013d). Another enzyme class responsible for degrading H_2O_2 are the PODs, which are capable of reducing H_2O_2 to H_2O . Guaiacol peroxidase is present in vacuoles, the cell wall, cytosol and extracellular spaces. POD is considered to be a marker of heavy metal toxicity, having broad specificity for phenolic substrates and higher affinity for H_2O_2 than CAT (Radwan et al. 2010). Guaiacol peroxidase consumes H_2O_2 to generate phenoxy compounds that are polymerized to produce cell wall components such as lignin (Mishra et al. 2006; Pourrut et al. 2011b).

Enzymes of ascorbate–glutathione cycle, APX and GR, are located mainly in chloroplasts, other cellular organelles and the cytoplasm, where they are involved in controlling the cellular redox status, especially under heavy metals stress conditions (Singh et al. 2010). Ascorbic acid is a primary and secondary antioxidant. APX utilizes ascorbate to reduce H_2O_2 to H_2O and O_2 (Mittler 2002; Triantaphylidès and Havaux 2009). During this process, ascorbate is oxidized to monodehydroascorbate.

Table 2 The antioxidant enzyme systems different plants use to defend themselves against heavy-metal-induced ROS

Heavy metals	Enzymes	Plant species	References
Ag	SOD, CAT	<i>Potamogeton crispus</i>	Xu et al. (2010b)
Al	SOD, CAT, APX, GPOX	<i>Hordeum vulgare</i>	Achary et al. (2012)
	SOD, POD	<i>Hordeum vulgare</i>	Guo et al. (2007)
As	SOD, GR, SDH	<i>Aspergillus niger</i>	Mukherjee et al. (2010)
	SOD, POD, APX, CAT	<i>Zea mays</i> , <i>Vicia faba</i>	Duquesnoy et al. (2010)
	APX, MDHAR, DHAR, SOD, GST	<i>Typha latifolia</i>	Lyubenova and Schröder (2011)
Cd	SOD, POD, SOD, CAT	<i>Carassius auratus</i>	Chen et al. (2012)
	SOD, APX, GR	<i>Gracilaria dura</i>	Kumar et al. (2012)
	APX, MDHAR, DHAR, GR, GST	<i>Helianthus annuus</i>	Nehnevajova et al. (2012)
	SOD, CAT, APX, GR	<i>Solanum lycopersicum</i>	Cherif et al. (2011)
	APX, MDHAR, DHAR, SOD, GST	<i>Typha latifolia</i>	Lyubenova and Schröder (2011)
	SOD, APX, CAT, GR	<i>Brassica juncea</i>	Ahmad et al. (2011b)
	SOD, POD, CAT	<i>Medicago sativa</i>	Xu et al. (2010a)
	POD, CAT	<i>Amaranthus hybridus</i>	Zhang et al. (2010)
	GSH, GST	<i>Brassica juncea</i>	Szőllősi et al. (2009)
	SOD, POD	<i>Hordeum vulgare</i>	Guo et al. (2007)
Cr	GPX, APX, CAT, GR	<i>Zea mays</i>	Mallick et al. (2010)
	APX, SOD, POD	<i>Lycopersicum esculatum</i>	Nayek et al. (2010)
Cu	APX, MDHAR, DHAR, GR, GST	<i>Helianthus annuus</i>	Nehnevajova et al. (2012)
	SOD, CAT, APX	<i>Pisum sativum</i>	Turchi et al. (2012)
	SOD, APX, GR	<i>Sesbania drummondii</i>	Israr et al. (2011)
	GPX, CAT	<i>Phaseolus vulgaris</i>	Bouazizi et al. (2010)
	SOD, POD, CAT	<i>Vetiveria zizanioides</i>	Xu et al. (2009)
	SOD, POD, APX, CAT	<i>Withania somnifera</i>	Khatun et al. (2008)
	SOD, GPX, CAT	<i>Datura stramonium</i>	Boojar and Goodarzi (2007)
		<i>Malva sylvestris</i>	
		<i>Chenopodium ambrosioides</i>	
		<i>Hordeum vulgare</i>	Guo et al. (2007)
Ni	SOD, CAT, APX, GPOX, GR	<i>Brassica juncea</i>	Kanwar et al. (2012)
	SOD, APX, GR	<i>Sesbania drummondii</i>	Israr et al. (2011)
Pb	SOD	<i>Spinacia oleracea</i>	Wang et al. (2010)
	APX, MDHAR, DHAR, SOD, GST	<i>Typha latifolia</i>	Lyubenova and Schröder (2011)
	SOD, APX	<i>Sedum alfredii</i>	Gupta et al. (2010)
	SOD, GPX, APX, CAT, GR	<i>Najas indica</i>	Sing et al. (2010)
	SOD, APX, GR	<i>Sesbania drummondii</i>	Israr et al. (2011)
	APX, SOD, POD	<i>Lycopersicum esculatum</i>	Nayek et al. (2010)
	SOD, CAT, AsA	<i>Zea mays</i>	Gupta et al. (2009)
	APX, GR, GST	<i>Lathyrus sativus</i>	Brunet et al. (2009)
	CAT, APX	<i>Wolffia arrhiza</i>	Piotrowska et al. (2009)
	APX, SOD, POD	<i>Lycopersicum esculatum</i>	Nayek et al. (2010)
Zn	APX, MDHAR, DHAR, GR, GST	<i>Helianthus annuus</i>	Nehnevajova et al. (2012)
	SOD, CAT, APX	<i>Pisum sativum</i>	Turchi et al. (2012)
	SOD, CAT, APX, GR	<i>Solanum lycopersicum</i>	Cherif et al. (2011)
	SOD, APX, GR	<i>Sesbania drummondii</i>	Israr et al. (2011)
	SOD, POD, CAT	<i>Vetiveria zizanioides</i>	Xu et al. (2009)

SOD superoxide dismutase, APX ascorbate peroxidase, GPX guaiacol peroxidase, CAT catalase, GR glutathione reductase, AsA ascorbic acid, GSH glutathione, GST glutathione S-transferase, POD peroxidase, DHAR dehydroascorbate; reductase, MDHAR monodehydroascorbate reductase, ACOX acyl co-A oxidase, SDH succinate dehydrogenase

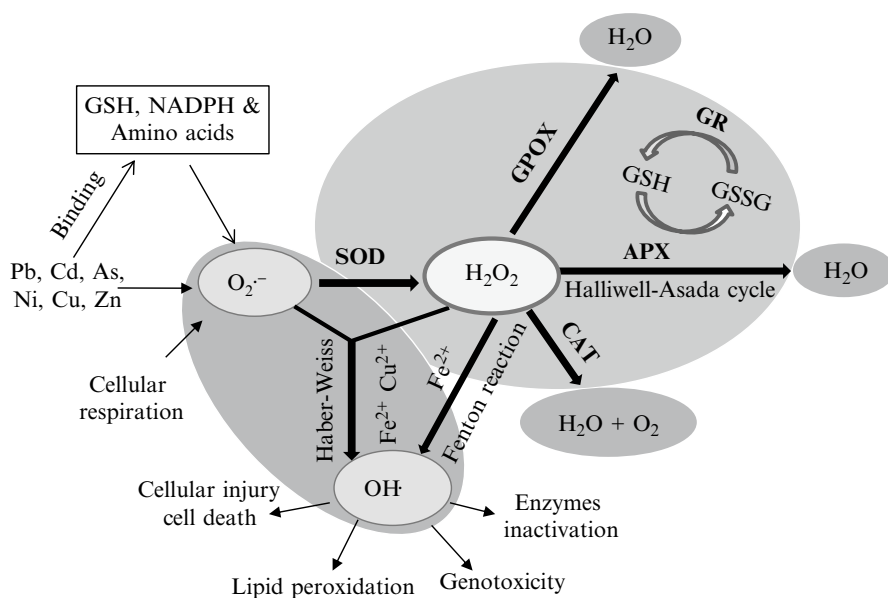


Fig. 4 Schematic representation of heavy-metal-induced oxidative stress. Under normal conditions (*highlighted grey*), $O_2^{\cdot-}$ is produced by cellular respiration. This $O_2^{\cdot-}$ is converted to H_2O_2 by SOD. The H_2O_2 produced is converted to H_2O and O_2 by the combined action of APX, GPOX, CAT and GR. In the presence of heavy metals, the $O_2^{\cdot-}$ and H_2O_2 production is increased. The increased ROS is incompletely converted to H_2O by the antioxidants. As a result, highly toxic HO^{\cdot} is produced by the Haber–Weiss or Fenton reactions. This HO^{\cdot} is the most toxic ROS and is believed to initiate lipid peroxidation, cell death, enzyme inactivation and genotoxicity

The monodehydroascorbate formed can be directly reduced back to ascorbate by monodehydroascorbate reductase (MDHAR), or may first be converted to dehydroascorbate, and then reduced by dehydroascorbate reductase (DHAR). In the process, GSH acts as reductant, which is oxidized to GSSG (oxidized glutathione). When GR activity is induced, the GSH/GSSG ratio remains high, and thus allows GSH to participate in PC synthesis and ROS detoxification (Noctor et al. 1998).

Several previous authors have reported heavy-metal-induced increases in antioxidant enzymes (Table 2). Ali et al. (2011) observed activation of SOD, POD, APX, GR and CAT under Al or Cr stress in *Hordeum vulgare*. Israr et al. (2011) reported a significant increase in enzymatic (SOD, APX, GR) antioxidant levels in *Sesbania drummondii* seedlings, when the seedlings were exposed to Cu, Ni and Zn alone and in combination. Lomonte et al. (2010) reported increased CAT and SOD activity, in response to applying Hg to *Atriplex codonocarpa* for 4 weeks under hydroponic conditions. Radić et al. (2010) also reported increased SOD and POD activity, when *Lemna minor* plants were exposed to Al and Zn. Yadav (2010) observed that the antioxidants CAT, APX and glutathione S-transferase (GST) increased as the Cr concentration increased in *Jatropha curcas*. Shahid (2010) reported a Pb-induced increase in APX, SOD, GPX and GR levels in *Vicia faba* roots and leaves, as did (Choudhary et al. 2010) in *Raphanus sativus* by Cu.

Increased activity of POD and CAT in *Amaranthus hybridus*, in response to Cd toxicity, was also observed by Zhang et al (2010). Singh et al. (2010) reported that the bioaccumulation of Pb by *Najas indica* activated several antioxidant enzymes (e.g., SOD, APX, GPX, CAT and GR). They also reported significantly increased cysteine synthase and glutathione-S-transferase activity. Similar results have been reported for *Phaseolus aureus* and *Vicia sativa* (Zhang et al. 2009). Recently, Shahid (2010) reported the results of a time course experiment (1, 4, 8; 12 and 24 h), in which the Pb-induced activation of antioxidant enzymes (APX, GPOX, SOD and GR), lipid peroxidation and ROS production occurred, after the Pb concentration reached significant levels in roots (after 1 h) and leaves (after 8 h). This suggests that Pb-induced lipid peroxidation, activation of enzymes and production of H₂O₂ are very rapid phenomena. Moreover, the oxidative bursts in roots and leaves coincide with periods of high Pb entrance rates to these tissues (1 and 12 h) (Pourrut et al. 2008).

7 Conclusions and Perspectives

In this review, we have highlighted key results from the previous and particular the recent published literature that addresses heavy-metal-induced physiological changes that occur in plants. Based on the literature cited in this review, we have drawn the following conclusions:

1. The generation of ROS is an inevitable feature of higher plants and other aerobic organisms. These ROS are constantly generated as side-products of certain metabolic pathways, and act to control various essential plant processes. Heavy metal exposure to plants disturbs the delicate balance between ROS production and elimination, leading to an enhanced steady-state ROS level that is called “**oxidative stress**”. A common feature of oxidative stress is damage to proteins, DNA, and lipids. Consequently, it is suggested that metal-induced oxidative stress in cells may partially be responsible for the toxic effects produced by heavy metals.
2. The plant kingdom has evolved a very efficient enzymatic and nonenzymatic defense system that allows ROS-scavenging to protect plant cells from oxidative damage. Retention of heavy metals in the cell wall is the first barrier against heavy metal stress. Heavy metal chelation by PCs, MTs, GSH and amino acids, and subsequent sequestration in vacuoles is another detoxification mechanism in plants. Biochemical tolerance to heavy metals is linked to activation of antioxidant enzymes. These heavy metal tolerance mechanisms may be activated separately or simultaneously, depending on the type and species of metal and plant.
3. ROS-induced toxicity to different plant molecules and the various responses of plants to over production of ROS are often used as bioindicators in risk and environmental quality assessment studies. Such biomarkers are appropriate for use in ecotoxicological studies. To further develop and improve these bioindicators, a better understanding of the processes and mechanisms involved in ROS production, their toxicity and defense mechanisms in the presence of pollutants, such as

heavy metals, are needed. Moreover, all bioindicators are not equally sensitive to different pollutants under different environmental conditions. Therefore, the mechanisms behind ROS production, toxicity and detoxification should be compared to optimize the most sensitive and efficient assays, with respect to environmental conditions like applied metal form and concentration, physico-chemical parameters of medium and metal and plant type.

8 Summary

As a result of the industrial revolution, anthropogenic activities have enhanced the redistribution of many toxic heavy metals from the earth's crust to different environmental compartments. Environmental pollution by toxic heavy metals is increasing worldwide, and poses a rising threat to both the environment and to human health. Plants are exposed to heavy metals from various sources: mining and refining of ores, fertilizer and pesticide applications, battery chemicals, disposal of solid wastes (including sewage sludge), irrigation with wastewater, vehicular exhaust emissions and adjacent industrial activity.

Heavy metals induce various morphological, physiological, and biochemical dysfunctions in plants, either directly or indirectly, and cause various damaging effects. The most frequently documented and earliest consequence of heavy metal toxicity in plants cells is the overproduction of ROS. Unlike redox-active metals such as iron and copper, heavy metals (e.g. Pb, Cd, Ni, Al, Mn and Zn) cannot generate ROS directly by participating in biological redox reactions such as Haber-Weiss/Fenton reactions. However, these metals induce ROS generation via different indirect mechanisms, such as stimulating the activity of NADPH oxidases, displacing essential cations from specific binding sites of enzymes and inhibiting enzymatic activities from their affinity for -SH groups on the enzyme.

Under normal conditions, ROS play several essential roles in regulating the expression of different genes. Reactive oxygen species control numerous processes like the cell cycle, plant growth, abiotic stress responses, systemic signalling, programmed cell death, pathogen defence and development. Enhanced generation of these species from heavy metal toxicity deteriorates the intrinsic antioxidant defense system of cells, and causes oxidative stress. Cells with oxidative stress display various chemical, biological and physiological toxic symptoms as a result of the interaction between ROS and biomolecules. Heavy-metal-induced ROS cause lipid peroxidation, membrane dismantling and damage to DNA, protein and carbohydrates. Plants have very well-organized defense systems, consisting of enzymatic and non-enzymatic antioxidant processes. The primary defense mechanism for heavy metal detoxification is the reduced absorption of these metals into plants or their sequestration in root cells. Secondary heavy metal tolerance mechanisms include activation of antioxidant enzymes and the binding of heavy metals by phytochelatin, glutathione and amino acids. These defense systems work in combination to manage the cascades of oxidative stress and to defend plant cells from the toxic effects of ROS.

In this review, we summarized the biochemical processes involved in the overproduction of ROS as an aftermath to heavy metal exposure. We also described the ROS scavenging process that is associated with the antioxidant defense machinery. Despite considerable progress in understanding the biochemistry of ROS overproduction and scavenging, we still lack in-depth studies on the parameters associated with heavy metal exclusion and tolerance capacity of plants. For example, data about the role of glutathione–glutaredoxin–thioredoxin system in ROS detoxification in plant cells are scarce. Moreover, how ROS mediate glutathionylation (redox signaling) is still not completely understood. Similarly, induction of glutathione and phytochelatins under oxidative stress is very well reported, but it is still unexplained that some studied compounds are not involved in the detoxification mechanisms. Moreover, although the role of metal transporters and gene expression is well established for a few metals and plants, much more research is needed. Eventually, when results for more metals and plants are available, the mechanism of the biochemical and genetic basis of heavy metal detoxification in plants will be better understood. Moreover, by using recently developed genetic and biotechnological tools it may be possible to produce plants that have traits desirable for imparting heavy metal tolerance.

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